EXHIBIT A

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Comparison of Metastatic Properties of a Variety of Mouse, Rat, and Human Cells in Assays in Nude Mice and Chick Embryos

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Abstract. *We have previously developed an assay to measure experimental metastatic ability of cells following intravenous injection into chorioallantoic membrane (CAM) veins of naturally immune deficient chick embryos. Here we compare metastatic properties of different cell types (*ras*-transformed and control NIH 3T3, LTA, and 10T1/2; melanoma; and glioma) from several species (mouse, rat, human), using chick embryos and the more commonly-used immune deficient host, nude mice. We found a good correlation between the two assays. Both hosts have advantages and disadvantages in assessing metastatic properties. We conclude that the chick embryo assay is a useful alternative host for experimental metastasis studies. This assay correlates well with and is less costly than assays using nude mice.*

Tumor metastasis frequently contributes to failure of cancer treatment. Because the spread of cancer cells from primary tumor to distant sites is a complex *in vivo* process, *in vivo* assays are required to study mechanisms of metastasis. We have developed an experimental metastasis assay in a naturally immune-deficient host, the chick embryo (1-4). In this assay, cells are injected intravenously into chorioallantoic membrane (CAM) veins of 11 day old embryos, and growth of tumor cells in embryonic organs is measured. In many ways this assay is analogous to the experimental (i.v.) metastasis assay in nude mice (5, 6).

In order to validate use of this metastasis assay in chick embryos, we have compared the metastatic properties of several cell lines when tested in assays in both chick embryos and nude mice. We have previously reported that T24 H-*ras*-transformed murine NIH 3T3 cells selected for increased metastatic ability in the chick assay (7, 8) also show increased metastatic ability in the nude mouse assay (9). Here we extend these results to two other murine cell lines (10T1/2 and LTA cells) also transfected with the H-*ras*-oncogene. In addition, we present data for other cell types (rat glioma, and human and murine melanoma) whose metastatic properties were compared using these two assays. Our results demonstrate that the chick embryo metastasis assay provides a convenient procedure for selection of cells that are also metastatic in nude mice, and that metastatic properties of a variety of cells from several species are similar when tested in these two hosts. These findings suggest that this assay may be a useful tool in studies of metastatic properties of cells.

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Key Words: Metastasis assay, chick embryo, nude mouse, *ras* oncogene, melanoma, glioma.

Materials and Methods

Cells and cell culture. Murine NIH 3T3 cells and a series of T24 H-*ras*-transformed NIH 3T3 cell lines (low p21-expressing C2PO, C5PO; high p21-expressing C2P2, C5P2, PAPO, PAP2) were derived and grown as described (8, 9), in Dulbecco's Minimal Essential Medium (DMEM; Gibco, Burlington, Ontario, Canada) plus 10% calf serum (Gibco). H-*ras* transformed (CIRAS-3) and control murine 10T1/2 cells were a gift from Dr. A.H. Greenberg (University of Manitoba) (10), and were grown in DMEM plus 10% calf serum, supplemented (for CIRAS-3) with G418 as described (10). LTA cells and *ras*- plus *myc*-transformed LTA cells were obtained and grown as described (2, 11).

Human melanoma cells (IGR39, IGR37) (12) were a gift from Dr. S.K. Liao (McMaster University, Hamilton, Ontario), and were grown in α-Minimal Essential Medium (without nucleosides) (Gibco) and 10% fetal calf serum. Murine B16F1 and B16F10 melanoma cells (13, 14) were a gift from Dr. I.J. Fidler (MD Anderson Cancer Center, Houston, TX). B16F1-M6 cells were isolated from a single metastatic liver focus following i.v. injection into chick embryos (Chambers et al., unpublished), and are likely clonal in origin (15). B16 melanoma cell lines were grown in α-Minimal Essential Medium with added nucleosides (Gibco) and 10% fetal calf serum.

Rat glioma C6 cells (16) were obtained from the American Type Culture Collection (CCL 107), and were grown in Ham's F10 medium with 15% horse serum and 2.5% fetal calf serum. Rat glioma P635 cells (17) were a gift from Dr. D. Bigner (Duke University), and were grown in the medium (above) used for C6 cells. P635-c45 is a glial filament negative clone which we derived from glial filament positive parental P635 cells (Chambers et al., manuscript submitted).

Experimental metastasis assay in chick embryos. Specified number of cells were injected intravenously into CAM veins of 11 day old embryos, in a volume of 0.1 ml/ embryo (1-4). Metastatic ability of the injected cells was

determined 7 days after injection by either histological sectioning of embryonic livers and examination for metastatic foci (for human cell lines) (2), or by means of the ouabain plating assay (for murine and rat cell lines). The ouabain plating assay, described previously (1-4), makes use of the natural resistance of rodent cell lines to ouabain cytotoxicity, relative to chick cells. Chick livers are dissociated into single cell suspensions, which are plated in 2 x 10^{-5} M ouabain, a concentration sufficient to kill chick cells while not affecting the plating efficiency or growth of rodent cells (1, 2, 4). Colonies that grow *in vitro*, following a 10-14 day incubation period, are counted and the numbers of viable rodent cells that had been present in the chick liver are calculated. We have found that metastatic cells grow in chick livers (and other organs) after i.v. injection, while normal cells and some tumorigenic cells do not. We have discussed details of this assay in greater detail elsewhere (1-4).

Experimental metastatic ability in nude mice. Specified numbers of cells were injected intravenously into tail veins of nude mice (HSD *nu/nu*, male, 4-6 weeks old) (5, 6). Mice were killed after 19-21 days (3 months for LTA-derived cell lines) and numbers of tumors in lungs and other organs were counted. Metastatic properties of B16 melanoma cell lines were assessed using syngeneic C57BL/6 mice (13, 14). Animals were cared for according to standards of the Canadian Council for Animal Care, under a protocol approved by the University of Western Ontario Council for Animal Care.

Results

Metastatic properties of ras-transformed cells. We previously showed that T24 H-*ras*-transformed NIH 3T3 cells could be selected *in vivo* for increased metastatic ability after i.v. injection into chick embryos, and that the metastatic populations expressed increased levels of *ras* (7, 8). We tested the experimental metastatic ability of the chick-selected cells in nude mice (9). The results of these two studies are summarized in Table I. We found that *ras*-transformed NIH 3T3 cells selected for metastatic ability in chick embryos (C2P2, C5P2, PAPO, and PAP2) are also metastatic in nude mice. These four cell lines express high levels of *ras*-encoded p21 protein and *ras* mRNA (8, 9, 18). C2PO and C5PO are *ras*-transformed clones that express little p21 protein (8, 9), and are non-metastatic in both chick embryos (8) and nude mice (9).

Table I. *Experimental metastatic ability of a series of* ras *transformed, murine NIH 3T3 cells.*

	Metastatic ability	
	in chick embryos[a]	in nude mice[b]
Non-metastatic		
NIH3T3	24 (0-104)	0 (0-0)
C2PO	91 (0-520)	0 (0-0)
C5PO	72 (13-470)	1 (0-8)
Metastatic		
PAPO	3.2×10^3 ($34\text{-}2\times10^4$)	3 (0-44)
PAP2	1.3×10^4 ($2\times10^3\text{-}1.6\times10^4$)	8 (0-50)
C2P2	2.2×10^3 ($1.1\times10^3\text{-}1\times10^4$)	4 (0-20)
C5P2	3.4×10^3 ($504\text{-}1.8\times10^4$)	20 (10-80)

C2PO and C5PO are clonal *ras* transformed cells that express little p21 or *ras* mRNA. C2P2 was selected from C2PO after two *in vivo* passages in the chick embryo; C5P2 was selected similarly from C5PO. C2P2, C5P2, PAPO, & PAP2 all express higher levels of *ras* (8,9). Numbers are medians (range).

a. Chick embryos: 10^5 cells/embryo were injected i.v. in CAM veins, and numbers of viable tumor cells per chick liver determined 7 days later, with the ouabain plating assay.

b. Nude mice: 5×10^4 cells/mouse were injected i.v. (tail vein), and numbers of lung tumors counted 19-21 days later.

Table II shows the results of a similar comparison of normal murine 10T1/2 cells, which are non-metastatic in both nude mice and chick embryos, and *ras*-transformed 10T1/2 cells (CIRAS-3) which are metastatic in both hosts. Again, metastatic properties in the chick embryo assay predicted metastatic behavior in nude mice.

Finally, we tested the metastatic properties of murine LTA cells, cells that we have identified as being resistant to both *ras*- and *myc*-induced progression (11, and Tuck et al., manuscript submitted). While LTA cells are *tumorigenic* in nude mice, they are *non*-metastatic in both chick embryos (2) and nude mice (Table III). This non-metastatic phenotype of LTA cells in both hosts is independent of expression of either *ras* or *myc* (Table III).

Metastatic properties of human and murine melanoma cells. We also tested human and murine melanoma cell lines for metastatic ability in both hosts (Table IV). Because human cells are more sensitive to ouabain cytotoxicity than are chick cells (2), the ouabain plating assay cannot be used to recover human cells from embryonic organs. Qualitative histological assessment of metastatic lesions was thus used for human cells tested in chick embryos (2). Human IGR37 cells are metastatic in both chick embryos and nude mice, while human IGR39 cells are not metastatic in either host (Table IV).

The metastatic ability of a series of murine B16 melanoma cell lines was tested in chick embryos and syngeneic mice (Table IV). B16F1 and B16F10 cells have similar metastatic properties in the chick assay, and grow in both chick livers (Table IV) and lungs (data not shown). In contrast, it has been found that B16F10 cells are more metastatic in mouse lungs than are B16F1 cells (13, 14). We confirmed these results in mice (Table IV). However, a cell line (B16F1-M6) which we selected from a metastatic lesion in chick liver following i.v. injection shows increased metastatic ability in both chick embryos and syngeneic mice, suggesting again that selection in the chick embryo can produce cells that show altered properties in mice. We do not yet fully understand all of the factors than can influence metastatic properties of B16 melanoma cell lines in chick embryos vs. syngeneic mice.

Metastatic properties of rat glioma cells. We also tested rat glioma cell lines (C6; P635, and P635-c45) for experimental metastatic properties in both chick embryos and nude mice (Table V). All of these cell lines were tumorigenic in nude mice (Chambers et al., manuscript submitted; and unpublished observations). All three of the lines were metastatic after i.v. injection in both chick embryos and nude mice

Table II. *Experimental metastatic ability of control and* ras*-transformed (CIRAS-3), murine 10T1/2 cells.*

	Metastatic ability	
	in chick embryos[a]	in nude mice[b]
10T1/2	48 (0-89)	0 (0- 0)
CIRAS-3	1.3×10^4 (5.2×10^3-1.5×10^4)	20 (1-65)

Numbers are medians (range).
a. Chick embryos: 10^5 cells/embryo were injected i.v. in CAM veins, and numbers of viable tumor cells per chick liver determined 7 days later, with the ouabain plating assay.
b. Nude mice: 5×10^5 cells/mouse were injected i.v. (tail vein), and numbers of lung tumors counted 19-21 days later.

Table III. *Experimental metastatic ability of murine LTA cells and LTA cells transfected with, and expressing, T24 H-*ras *and v-*myc *oncogenes.*

	Metastatic ability	
	in chick embryos[a]	in nude mice[b]
LTA	7 (0-44)	0 (0-0)
LTA-*ras*	0 (0-80)	0 (0-0)
LTA-*ras*+*myc*	0 (0-30)	0 (0-0)

Numbers are medians (range).
a. Chick embryos: 5×10^5 cells/embryo were injected i.v. in CAM veins, and numbers of viable tumor cells per chick liver determined 7 days later, with the ouabain plating assay.
b. Nude mice: 5×10^5 cells/mouse were injected i.v. (tail vein), and lungs were examined for tumor formation after 3 months.

Table IV. *Experimental metastatic ability of human and murine melanoma cells.*

	Metastatic ability	
	in chick embryos[a]	in mice[b]
Human melanoma		
IGR37	++	8 (0- 11)
IGR39	--	0 (0- 0)
Murine melanoma		
B16F1	5×10^4 (1×10^4-8×10^4)	24 (0- 40)
B16F10	1.4×10^4 (3.2×10^3-4.2×10^4)	65 (10-110)
B16F1-M6	1.5×10^5 (8×10^4-2.6×10^5)	55 (12-200)

Numbers are medians (range).
a. Chick embryos: 10^5 cells (for IGR cells) or 5×10^3 cells (for B16 cells) per embryo were injected i.v. in CAM veins. Metastatic ability of IGR cells in chick embryo livers was assessed qualititively from histological sections (2), 7 days after injection; ++ indicates metastases were detected;– indicates metastases were not detected. Metastatic ability of B16 cells was determined with the ouabain plating assay, also after 7 days.
b. Mice: *Nude mice* were used for testing human IGR37 & IGR39 cells, which were injected i.v. (tail vein) at 5×10^4 cells/mouse, and numbers of lung tumors counted 19-21 days later. *Syngeneic* C57BL/6 mice were used for testing murine B16 cells, which were injected i.v. at 5×10^4 cells/mouse, as above.

Table V. *Experimental metastatic ability of rat glioma cell lines.*

	Metastatic ability	
	in chick embryos[a]	in nude mice[b]
P635	1.3×10^5 (2×10^4-2.4×10^5)	77 (48-121)
P635-c45	1.4×10^5 (1×10^5-1×10^6)	101 (28-214)
C6	5.2×10^3 (3.1×10^3-1.1×10^4)	9 (1- 13)

Numbers are medians (range).
a. Chick embryos: 10^4 cells/embryo were injected i.v. in CAM veins, and numbers of viable tumor cells per chick liver determined 7 days later, with the ouabain plating assay.
b. Nude mice: 10^4 cells/mouse were injected i.v. (tail vein), and numbers of lung tumors counted 19-21 days later.

(Table V), again confirming that metastatic properties in the chick embryo predict metastatic properties in nude mice.

Discussion

The complexity of the metastatic process demands a variety of assays for its effective study. We have previously discussed the range of assays that are available for studying aspects of tumor metastasis (3, 4, 19), including *in vitro* assays (e.g., for invasive ability, or protease activity), "experimental" metastasis assays (formation of tumor foci in internal organs after intravenous injection of tumor cells), and "spontaneous" metastasis assays (formation of tumor foci in internal organs following growth of an artificial primary tumor after intramuscular or subcutaneous injection of tumor cells). The most commonly used animal host for *in vivo* metastasis assays is the laboratory mouse. Many assays require use of an immune deficient host, often the athymic nude mouse.

There are a number of reasons for consideration of alternatives to nude mice for these assays. First, the current climate of concern for the care of animals used in tumor experimentation (20) encourages use of alternative assays. There is often less objection to the use of avian embryos than there is to the use of laboratory mice. It must be stressed that the metastasis assay described here, using chick embryos, cannot be used solely and instead of assays in mice. However, use of the chick embryo assay may in some cases reduce the numbers of mice required for these studies. The complexity of the metastasis process, coupled with the lack of suitable *in vitro* assays for all steps and our poor understanding of how all the steps in metastasis interact, necessitates the use of *in vivo* assays for metastasis.

Second, the commonly used host for *in vivo* assays, the nude mouse, is expensive and requires special care and housing. We have previously calculated that the cost of testing experimental metastatic properties of a series of cell lines, using the chick assay described here, is approximately one-quarter the cost of testing the same cells in nude mice (with an endpoint of counting metastatic tumors), and about one-fifth the cost of testing in nude mice and plating mouse lungs to recover viable cells (9). Furthermore, our calculations did not include "start-up" costs, i.e. facilities for hous-

ing nude mice, which are expensive, vs. facilities for maintaining chick embryos, which are inexpensive (9). Because of these costs, the use of nude mice for metastasis studies is an option that may simply be unavailable to some scientists. The assay described here, using the more accessible chick ebryo, may be a useful option in these cases. In addition, scientists who do have access to nude mouse facilities may find the chick embryo assay useful as a cost-saving pre-screen to identify cell lines of interest, which then may be tested in nude mice.

We have summarized here our experience with a variety of cell lines, from several species, when tested in experimental, intravenous metastasis assays in chick ebryos vs. nude mice. Obviously, if the chick embryo is to be a useful adjunct to nude mouse assays, the results obtained in the two assays should show a general correlation. From the results presented in Tables I-III, we conclude that the metastatic properties of a variety of *ras*-transformed murine cells can be adequately assessed by experimental metastasis assays in both chick embryos and nude mice. *Ras*-transformed cells selected for increased metastatic ability in the chick also show increased metastatic ability in nude mice.

In addition, we tested a series of melanoma (Table IV) and glioma (Table V) cells in both hosts. We found that the majority of cell lines tested show similar metastatic properties in the two assays. A notable exception was the inability of the chick assay to distinguish between murine B16F1 and B16F10 cell lines (Table IV), while the same cell lines tested in syngeneic mice generally behave as poorly (B16F1) and highly (B16F10) metastatic cells, in terms of numbers of tumor foci formed in mouse lungs after intravenous injection (13, 14). We do not yet understand the difference detected between the assays by these two cell lines. However, a cell line (B16F1-M6; Table IV) which we selected following intravenous injection of B16F1 cells from a metastatic lesion in chick embryo chick liver, showed increased metastatic ability when tested in both chick embryos and syngeneic mice, suggesting that selection for metastatic growth ability in the chick embryo can produce cells with increased metastatic ability in mice. For all other cell lines tested (*ras*-transformed NIH 3T3, 10T1/2, and LTA cell lines; human melanoma cell lines; rat glioma cells lines), we found that the cells defined as metastatic by the chick embryo assay were also metastatic when tested in nude mice.

Two disadvantages to the chick embryo assay should be considered. First, tumorigenicity (formation of a local tumor after intramuscular or subcutaneous injection) and subsequent spontaneous metastasis from that tumor, are perhaps better tested in assays in mice. This sort of assay has been used with success by some groups using the chick embryo (21, 22), but in our experience the creation of "primary" tumors on the CAM surface of the chick embryo is subject to large variability (1), which depends in part on damage induced on the CAM surface during experimental manipulation (1, 23). For experiments that require information on spontaneous metastatic ability, assays in mice continue to be preferable. Second, the ouabain plating assay cannot be used to recover and quantify *human* cells in the chick embryo assay (2). Human cells are considerably more sensitive to ouabain cytotoxicity than are chick cells (2), and thus cannot be recovered by this procedure the way rodent cells can. Human tumor cell growth can be detected in chick organs histologically (2), or other procedures can be devised for the recovery of human cells (i.e., use of human cells transfected with a drug resistance marker such as neo^R). The extent of species difference in ouabain cytotoxicity between chick and rodent cells (1, 2), however, means that any *rodent* cell line can be recovered quantitatively from chick organs after intravenous injection, using the ouabain plating assay.

From our previous work using this assay (1-4, 7-9, 11, 15, 24), as well as results presented here, we conclude that the chick embryo experimental metastasis assay is an efficient, cost-effective procedure for screening cells for metastatic properties in an immune-deficient host. The assay is rapid (7 days *in vivo,* plus 10-14 days *in vitro* for most cells). It also can provide kinetic information on tumor cell growth *in vivo* (1-3, 7, 8, 24), which is not obtained from assays in mice with an end-point of counting tumor foci. We have found this assay to be useful for *in vivo* selection of cells that are also metastatic in nude mice (8, 9) and find that experimental metastatic properties of cells in the chick embryo often predict their behavior in nude mice.

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